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Effects of microbial transglutaminase treatment on physiochemical properties and emulsifying functionality of faba bean protein isolate

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Abbreviations: CDs, conjugated dienes; EAI, emulsifying activity index; FBPI, faba bean protein isolate; FI, fluorescence intensity; FUCD, far-ultraviolet circular dichroism; MTG, microbial transglutaminase; OPs, protein oxidation products; O/W, oil-in-water; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abstract

The potential use of microbial transglutaminase (MTG)-treated faba bean protein isolate (FBPI) as emulsifiers to maintain physical and oxidative stability of oil-in-water (O/W) emulsion was investigated. MTG-treated FBPIs (MTG-FBPIs) were prepared by incubating with MTG for 60, 120 or 240 min. O/W emulsions were stabilized by 3% (w/v) of MTG-FBPIs or control-FBPI (treated with inactive MTG) and stored at 37 °C for 7 days. MTG treatments induced cross-linking in FBPI, raised the protein net surface charges by 5%–8%, and increased the emulsion particle size by 19%–135%. MTG treatment for 120 and 240 min but not 60 min induced excessive surface hydrophobicity, resulting in decreased emulsifying activity and physical stability of emulsion. By day 7, all MTG-treated FBPIs showed similar inhibiting effects against lipid oxidation in emulsion, indicated by less conjugated dienes and hexanal production. MTG-FBPIs moderately promoted protein oxidation (120 min > 240 min ≈ 60 min). Thus, prolonged MTG treatment should be avoided to prevent accelerated protein oxidation and droplets coalescence. MTG treatment for 60 min makes FBPI a potential emulsifier to maintain physical stability while improving lipid oxidative stability in emulsion, potentially attributed to thicker interfacial layer, larger droplet size, and protective effect of protein.

Keywords:

Faba bean protein, microbial transglutaminase, emulsifying property, emulsion, oxidative stability

1. Introduction

Faba bean (*Vicia faba* L.) is a promising alternative protein source to animal and soybean in that it is rich in protein (27 to 34% of the dry weight), widely grown due to easy cultivation, and sustainable (Karatas, Gunay, & Sayar, 2017). Moreover, faba bean protein has many functional properties such as water and fat binding, foaming, and gelation capacities (Boye, Zare, & Pletch, 2010). The potential use of faba bean protein isolate (FBPI) as an emulsifier has been reported (Gumus, Decker, & McClements, 2017a, 2017b, 2017c). Meanwhile, microbial transglutaminase (MTG) treatment has been shown to improve the emulsifying properties of soy proteins by creating inter-molecular cross-linking (Babiker, 2000). However, it is not clear whether MTG improves the emulsifying activity of FBPI.

MTG is a commercial type of food-grade transglutaminase (γ-glutaminyl-peptide, amine γ-glutamyltransferase, EC 2.3.2.13). It catalyzes acyl transfer reactions between γ-carboxyamide of peptide or protein-bound glutamyl residue (acyl donor) and primary amino group (acyl acceptor). When lysine residues act as acyl acceptor, ε-(γ-glutamyl)-lysine "isopeptide" covalent bonds are formed, resulting in polymerization or amine incorporation (Luisa, Gaspar, & de Goes-Favoni, 2015). MTG treatment improves the thermal and pH stability of whey protein isolate (Agyare & Damodaran, 2010; Damodaran & Agyare, 2013).

MTG treatment on milk protein induced moderate cross-linking, thereby improving the physical stability in emulsions (Faergemand, Otte, & Qvist, 1998). Apart from physical disturbance, emulsions are also susceptible to oxidative deterioration. Approaches are taken to inhibit lipid oxidation in emulsions. For example, the animal protein and its hydrolysates inhibit lipid oxidation in emulsion at the expense of protein oxidation (Intarasirisawat, Benjakul, Visessanguan, & Wu, 2014; Yang & Xiong, 2018). However, protein oxidation not only causes the loss in quality and nutritional value, but also contributes to a variety of diseases (Estevez &

Luna, 2017). Therefore, besides physical stability, it is also important to manage the oxidative stability of both lipid and protein.

Therefore, the objectives of this study is to (1) investigate how MTG treatment affects the physiochemical properties and emulsifying activity of FBPI, (2) examine whether the MGT-modified FBPI improves the physical and oxidative stability of lipid and protein in O/W emulsions.

2. Materials and methods

2.1. Materials

Faba beans (cultivar “Divine 2012”) were grown at Viikki Experimental Farm of the University of Helsinki in Finland. MTG (ACTIVA®-WM, with 99% maltodextrin) with approximately 80 U/g activity was donated by Ajinomoto Food Europe S.A.S. (Mesnil-Saint-Nicaise, France). Rapeseed oil was purchased from a local store. Bovine serum albumin (BSA) and linoleic acid (≥99%) were obtained from Sigma-Aldrich (Steinheim, Germany). Tocopherol standards (α-, β-, γ-, δ-tocopherol), sodium hydroxide (NaOH), di-sodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate and sodium azide were purchased from Merck (Darmstadt, Germany). All other chemicals employed in this study were of analytical grade.

2.2. Extraction of FBPI

Faba beans were dehulled and milled. Fine faba bean flours with an even particle size were obtained with the high speed rotor ultra-centrifugal mill (sieve pore size 0.5 mm, Ultra Centrifugal Mill ZM 200, Retsch, Germany). FBPI was extracted by isoelectric precipitation (Damodaran & Kinsella, 1981). Briefly, faba bean flour was dissolved in water (flour: water

=1:10, w/v) adjusted to pH 8.0 with 2 N NaOH, and stirred at room temperature for 4 hours. After centrifugations, the supernatant was adjusted to pH 4.5 with 2 N HCl. The pellet collected after centrifugation was re-dissolved in water re-adjusted to pH 8.0 overnight. The acid precipitation and alkaline dissolution were repeated for three times. Afterwards, the pellet collected was dialyzed against water at pH 8.0 for 48 hours using a molecular porous membrane tubing (Spectra/Por® MWCO 6-8000, Spectrum Medical Devices, Rancho Dominguez, CA, USA), and then lyophilized on a freeze-dryer (FTS Systems Inc., Stone Ridgeny, NY, USA). The yield of FBPI was 88.6%, determined by the Biuret method. FBPI was stored at -20 °C for further use.

2.3. MTG treatment

MTG-treated FBPI (MTG-FBPI) was prepared as previously described with minor modifications (Damodaran & Agyare, 2013). FBPI dispersions in water were incubated with MTG (5 U/g of protein substrate) at 37 °C with constant stirring for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI). A control-FBPI was prepared by adding NH_4Cl-inactivated MTG (addition of NH_4Cl to a final concentration of 10 mM) to FBPI dispersions. FBPI dispersion without MTG was used as native-FBPI.

2.4. Effects of MTG treatment on FBPI

2.4.1. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was employed to examine FBPI changes (Agyare & Damodaran, 2010). A 4-20% gradient acrylamide separating gel and a 4% acrylamide stacking gel were used. Protein samples for SDS-PAGE were prepared by mixing 0.2% (w/v) of native FBPI, control-FBPI, and MTG-FBPIs with an equal-volume of sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.125 M Tris, pH 6.8), and then heated in boiling water for 3 min to

completely denature the samples. Next, 15 µL of prepared protein samples were loaded onto each lane of the gel. An EZ-Run™ Pre-Stained Protein Marker (20 kDa–118 kDa, Fisher BioReagents™, Vilnius, Lithuania) was loaded along with protein samples. The electrophoresis run on a Mini-PROTEN 3 apparatus (Bio-Rad laboratories, Hercules, CA, USA). The regression line calculated by plotting the log of standards molecular weight (MW) versus relative mobility (Rf) of the protein markers was used to estimate MW of unknown proteins.

2.4.2. Fluorescence measurement

The intrinsic fluorescence emission spectra of control and MTG-FBPIs were measured by a LS 55 Luminescence Spectrometer (PerkinElmer Inc., Waltham, MA, USA). Protein dispersions (0.05% protein solution, pH 7.0) were excited at 295 nm and emission intensity was measured from 310 to 400 nm. Slit width of 7 nm and speed of 180 nm/min were chosen.

2.4.3. Circular dichroism measurement

Far-ultraviolet circular dichroism (FUCD) of FBPIs (0.1% protein solution) were measured in 0.1 cm path length quartz cuvettes at 25 °C using a Jasco J- 815 CD spectrometer equipped with a Peltier controlled temperature accessory (Jasco International, Tokyo, Japan). Three scans of each sample were averaged and expressed as the mean ellipticity [θ] values. All the FUCD spectra were corrected by subtracting the spectrum of Milli-Q water.

2.4.4. Surface charge (Zeta potential)

The overall surface charges of FBPIs were determined by measuring the electrophoretic mobility (U_E) of protein dispersions (0.2%, w/v) at pH 7.0 using a Zetasizer Nano-ZS90 instrument (Malvern Instruments, Westborough, MA). U_E was used to calculate the zeta potential as follows:

$$U_E = \frac{2\varepsilon * \xi * f(\kappa\alpha)}{3\eta}$$

where ε is the permittivity, f(κα) is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity. The Smoluchowski approximation f(κα) equaled to 1.5.

2.4.5. Surface hydrophobicity

Surface hydrophobicity (S_0-ANS) of FBPIs were measured at pH 7.0 as previously described with minor modification (Karaca, Low, & Nickerson, 2011). FBPI dispersions were diluted with 10 mM sodium phosphate buffer (SPB, pH = 7.0) to give five gradient concentrations ranged from 0.005% to 0.025% (w/v). To 3.2 ml of these solution, 32 μl of the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS: 8 mM ANS solution in 10 mM SPB, pH 7.0) was added, followed by vortexing for 5 s. Samples were kept in the dark for 5 min. Fluorescence intensity (FI) was then measured by a LS 55 Luminescence Spectrometer (PerkinElmer Inc., Waltham, MA, USA) with excitation and emission wavelengths at 390 and 470 nm. Slit widths were set to 2.5 nm. The FI attributed to protein in the buffer was subtracted from the FI of each sample with ANS. The initial slope of the FI versus protein concentration was calculated by linear regression analysis to represent protein surface hydrophobicity.

2.4.6. Protein solubility

FBPI dispersions (1%, w/v) were stirred magnetically for 10 min at pH of 7.0. Then the dispersions were centrifuged at 12000 × g for 20 min at 20 °C. After appropriate dilution, the protein content of the supernatant was determined by the Biuret method. The protein solubility was expressed as percentage of supernatant protein over total protein.

2.5. Preparation of emulsions

Four different emulsions: Control, MTG60, MTG120, and MTG240 were prepared by Control-FBPI, MTG60-FBPI, MTG120-FBPI and MTG240-FBPI, respectively. The emulsions consisted of 3% (w/v) of control or MTG-FBPIs, and 10% (w/v) purified rapeseed oil. Briefly, a coarse emulsion was prepared by blending different FBPI dispersions and purified rapeseed oil for 2 min using an Ultra-Turrax® T25 homogenizer at 13500 rpm (IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Purified rapeseed oil was obtained by chromatographic purification as previously described (no detectable residual tocopherols by normal-phase HPLC with a fluorescence detector) (Lampi, Dimberg, & Kamal-Eldin, 1999). Then, a more stable emulsion was obtained by homogenizing the coarse emulsion by an M-110Y Microfluidizer® processor (Microfluidics™, MFIC Corp., Westwood, MA, USA) at an operating pressure of 600 bars for 10 minutes. Next, sodium azide was added to a final concentration of 0.02% (w/v) to inhibit microbial growth. Finally, the emulsion was equally divided into three sealed vials and stored at 37 °C in dark with constant magnetic stirring. Samples were collected on day 0, 1, 4 and 7 to determine the physical and chemical stability.

2.6. Emulsions properties

2.6.1. Droplet size

The initial size distribution of emulsion droplet was determined at room temperature after appropriate dilution on a laser light scattering instrument (Mastersizer 3000, Malvern Mastersizer, Malvern Instruments, Ltd., Worcestershire, UK) with a hydro EV dispersion unit. The average droplet diameter (d_{32}) of oil droplets in the emulsions was recorded.

2.6.2. Emulsifying activity index (EAI)

A 100 µl emulsion sample was immediately (0 min) removed from the bottom of the homogenized emulsion and added to 7.5 mL of 10 mM SPB (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS). After vortexing for 5 s, the absorbance of the diluted emulsion was

measured at 500 nm using a UV/Vis spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). EAI was calculated as follows:

$$EAI\ (m^2/g) = \frac{2 * 2.303 * A_0 * DF}{C * \emptyset * \theta * 10{,}000}$$

where DF is the dilution factor, C is the initial concentration of protein (g/mL), Ø is the optical path, θ is the fraction of oil used to form the emulsion and A_0 is the absorbance of the diluted emulsions at 0 min.

2.6.3. Morphology

A drop of emulsion was placed on a slide glass and covered with a cover glass. The microstructure of emulsions was observed at room temperature using an optical microscope (Axio Scope A1, Carl Zeiss Inc., Oberkochen, Germany) equipped with an AxioCam camera under × 100 objective.

2.7. Lipid oxidation

Lipid oxidation was evaluated by formation of conjugated dienes (CDs) and hexanal. CDs was measured as previously described with minor modifications (Estevez, Kylli, Puolanne, Kivikari, & Heinonen, 2008). Briefly, 100 µL of emulsion was dissolved with 1.5 mL of isooctane/isopropanol (2:1, v/v) and vortexed for 30 s vigorously. After centrifugation at 550 × g for 5 min at 4 °C, 200 µL of upper organic phase was collected, diluted and vortexed with 4.8 mL isooctane. Then, the absorbance was measured at 234 nm by a UV/Vis spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). The CDs concentration was calculated using 25200 $M^{-1}cm^{-1}$ as the molar extinction coefficient.

Hexanal was measured by headspace solid-phase micro extraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS) as described (Damerau, Kamlang-

ek, Moisio, Lampi, & Piironen, 2014). The system comprised of a HS-SPME injector (combiPAL, CTC Analytics, Lake Elmo, MN, USA) with a DVB/CAR/PDMS-fiber (50/30 μm film thickness; Supelco, Bellefonte, PA, USA), GC (HP 6890 series, Agilent Technologies Inc., Wilmington, DE, USA) equipped with a capillary column SPB-624 (30 m × 0.25 mm i.d., 1.4 μm film thickness; Supelco, Bellefonte, PA, USA) and MS detector (Agilent 5973 Network, Agilent Technologies Inc., Wilmington, DE, USA).

2.8. Protein oxidation

Protein oxidation was evaluated by monitoring the loss of tryptophan fluorescence and formation of protein oxidation products (OPs) (Estevez et al., 2008). Emulsion was firstly diluted to designed concentrations (1 μL/mL water for tryptophan fluorescence and 40 μL/mL water for OPs). Emission spectra of tryptophan fluorescence were recorded from 310 to 400 nm with an excitation wavelength of 295 nm. Emission spectra of OPs were recorded from 420 to 500 nm with the excitation wavelength at 360 nm. Slit width of 7 nm and speed of 180 nm/min were set for all the measurements. Tryptophan standards was used as quality control for the fluorescence measurements.

2.9. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical analysis was conducted on SAS 9.4 software (Cary, NC, USA). One-way analysis of variance (ANOVA) followed by Tukey's test for post hoc comparison was used to determine treatment effects among groups. To determine time-dependent changes within each treatment group, repeated measure (RM) One-way ANOVA with Tukey's test was used. The significant level was set at $\alpha = 0.05$ for all tests. All samples were prepared in triplicate.

3. Results and discussion

3.1. Effect of MTG on FBPI structure

MTG treatment induced cross-linking of FBPI. The native-FBPI had three main bands: MW of ~ 24 kDa, ~51 kDa, and ~ 42 kDa (**Fig. 1, lane 1**). These correspond to the α- and β- fractions of legumine-like 11S, and subunit of vicilin-like 7S (Wright & Boulter, 1972, 1974). The control-FBPI showed similar pattern with native-FBPI, indicating the inactivation of MTG was thorough and the amount of MTG did not affect the SDS-PAGE pattern (**Fig. 1, lane 2**). In MTG-FBPIs, new protein bands which corresponded to MW ~ 65 kDa, ~ 85 kDa and > 118 kDa appeared (**Fig. 1, lane 3-5**). The new protein species were presumably to be homo- and/or hetero- dimers and polymers of 11S and 7S by cross-linking (Damodaran & Agyare, 2013). In addition, new polymeric species (P1 and P2, > 118 kDa) were aggregated at the top of separating gel and stacking gel, which appeared to be polymers from cross-linking between monomers and intermediary subunits of 11S and 7S (Wright & Boulter, 1972, 1974).

The fluorescence properties of tryptophan are sensitive to the environment and are used to indicate the conformational change of protein (Agyare & Damodaran, 2010). The λ_{max} of tryptophan fluorescence shifted from ~330 to ~333 nm after MTG treatment (**Fig. 2A**). This red shift indicated more tryptophan residues were exposed to the aqueous environment (Eissa, Puhl, Kadla, & Khan, 2006). However, the fluorescence intensity did not significantly change after treatment. MTG-FBPI showed similar FUCD spectra to control (**Fig. 2B**). This indicated MTG did not induce apparent changes in the secondary structure. The FUCD spectra displayed a positive band (190 - 200 nm) and a negative band (210 - 225 nm), which are the characteristic features of β-sheet structure (Matsuura & Manning, 1994).

3.2. Physiochemical and emulsifying properties

The control-FBPI had a negative surface charge (~ -28.73 mV) (**Fig. 3A**). The surface charge values were comparable to previous report of faba bean protein (Karaca et al., 2011).MTG treatments decreased the potential by 5% - 8%. Surface charge is an important for protein to form and stabilize emulsions (Karaca, Low, & Nickerson, 2015). Once forming a physical interfacial layer around the oil droplet, the electrostatic charges of protein produce repulsive forces that prevent the coalescence (McClements, Bai, & Chung, 2017).

Surface hydrophobicity measures the relative percentage of hydrophobic groups exposed on protein surface (Karaca et al., 2015). MTG treatment for 120 and 240 min, but not 60 min increased the surface hydrophobicity by 13% and 8%, respectively (**Fig. 3B**). A balance of hydrophilic and hydrophobic groups makes proteins ideal emulsifiers. If the protein is too hydrophilic, it cannot strongly adsorb oil droplet surface; being too hydrophobic will reduce water solubility and surface activity (McClements et al., 2017). Protein solubility promotes greater migration of the oil-in-water emulsion interface (Karaca et al., 2015). Both control and MTG-FBPI showed similar solubility (**Fig. 3C**). The increased electrostatic repel might offset the effects of the cross-linking and increased hydrophobicity on solubility, thus MTG did not affect the solubility in this study.

EAI is an estimate of the interfacial area stabilized per gram protein based on turbidity of a diluted emulsion (Pearce & Kinsella, 1978). The control-FBPI and MTG60-FBPI had an EAI of ~ 40 m^2/g (**Fig. 3D**). This is similar to soy protein isolates (Karaca et al., 2011). By contrast, treatment for 120 and 240 min decreased the EAI by 17% and 39%, respectively. EAI is affected by various factors, including the physiochemical features discussed above. The decreased emulsifying activity in MTG120 and MTG240 emulsions may be partly explained by excessively increased surface hydrophobicity (**Fig. 3B**). Therefore, prolonged MTG treatment should be avoided to maintain the emulsifying activity of FBPI.

3.3. Physical properties of emulsions

MTG treatment increased the particle size of FBPI-stabilized emulsions (**Fig. 4**). The volume-surface mean particle diameter d_{32} of control and MTG-FBPI emulsions ranged from 0.06 to 0.15 μm. MTG treatment for 60, 120 and 240 mins increased the d_{32} values by 19%, 59%, and 135%, respectively. Larger particle size of emulsions suggested smaller surface area, which in turn might lower oxidation rate (McClements & Decker, 2000). On the other hand, bigger particle size might lower physical stability (Phoon, Paul, Burgner, San Martin-Gonzalez, & Narsimhan, 2014). In accordance with the d_{32} measurement, microscopy revealed that emulsions stabilized by MTG-FBPI had larger initial droplets than the control emulsion (**Fig. 5**). The control and MTG60 emulsion exhibited a more homogeneous distribution than MTG120 and MTG240 emulsion. After 7 days of storage, the control emulsion and MTG60 appeared stable while droplets coalescence was observed in MTG120 and MTG240 emulsions.

3.4. Oxidative stability

3.4.1. Lipid oxidation

CDs and hexanal were monitored as indicators of lipid oxidation (**Table 1**). Both CDs and hexanal levels continuously increased in all emulsions. On day 7, CDs in MTG60, MTG120 and MTG240 emulsions were 17%, 14%, and 14% lower than control emulsion. Similarly, hexanal in MTG60 and MTG120 was 65% and 62% lower than the control emulsion. However, hexanal in MTG240 did not differ from control emulsion. This indicated that MTG treatment on FBPI for 60 and 120 min, but not 240 min increased the oxidative stability of lipids in O/W emulsions. Similarly, MTG treatment on casein improved oxidative stability in flaxseed oil emulsion, and increased dose of MTG did not further improve the effects (Ma et al., 2012).

Several mechanisms may explain the improved lipid-oxidative stability by MTG treatment. Oxygen initiates lipid oxidation once it is transported into the oil phase, thus transport of oxygen across the interface is the rate-controlling step (McClements & Decker, 2000). Apart from oxygen, metal ion is the dominant prooxidant that influences oxidation in emulsions (McClements & Decker, 2000). By creating a protein barrier between aqueous metal ions and those in the lipid, the lipid oxidation would be limited (Ma et al., 2012; Tong, Sasaki, McClements, & Decker, 2000). In addition, a thicker layer might be formed as evidenced by increased emulsion particle size (Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012). MTG induces cross-linking at the side chains which creates branched polymers and steric constraint. The majority of the peptide segment of the branched polymers may not directly contact with the interface. As a result, a thick layer protruding from the surface of the oil droplet is formed (Liu & Damodaran, 1999). Presumably, this thicker layer inhibited the diffusion of oxygen and transition metal ions across the layer. Furthermore, the rate of lipid oxidation is inversely associated with particle size of emulsion due to the amount of contact area between lipid and aqueous phase (Ma et al., 2012; McClements & Decker, 2000). As the discussed above, emulsions stabilized by MTG-FBPI had larger particle size than the control emulsion, which also partially explained the inhibiting effects against lipid oxidation.

3.4.2. Protein oxidation

Decrease in tryptophan fluorescence indicates oxidative degradation of proteins in O/W emulsions (Estevez et al., 2008). On day 1, MTG120 and MTG240 emulsions started to show significant lower tryptophan fluorescence than the control emulsion (**Table 2)**. By contrast, MTG60 emulsion maintained similar level of tryptophan fluorescence with control emulsion till day 4. Protein oxidation is accompanied by the generation of a collection of various OPs, some in the form of carbonyls (Salminen & Heinonen, 2008). The MTG-FBPI

emulsions had similar level of OPs with control emulsions on day 1 and 4. On day 7, OPs in MTG60 and MTG120 were significantly higher than in the control emulsion (**Table 2**). Thus, when the storage time was extended, MTG-FBPIs did not improve the oxidative stability of proteins in the O/W emulsions.

To better understand why longer MTG treatment on FBPI appeared to accelerate protein oxidation, emission spectra of tryptophan fluorescence was recorded (**Fig. 6**). Fluorescence peak of tryptophan in MTG-FBPI emulsions had a red shift from ~ 330 nm to ~ 334 nm. The shift of λ_{max} suggested that tryptophan residues in MTG-FBPI emulsions changed to a relatively polar environment and were exposed more to the aqueous environment (Marcuse & Fredriks.Po, 1968). This is in agreement with the results of intrinsic emission fluorescence of FBPI dispersions (**Fig. 2A**). In addition, MTG treatment for 120 and 240 mins significantly increased the surface hydrophobicity of FBPI (**Fig. 3B**). Increased surface hydrophobicity suggests the exposure of nonpolar amino acids previously embedded inside the protein structure. Similarly, MTG treatment produced a higher degree of unfolding with increased exposure of oxidizable hydrophobic residues to the solvent, resulting in a less compact structure of protein (Baez, Moro, Ballerini, Busti, & Delorenzi, 2011). Furthermore, positively charged interfacial layer could repel cationic metal ions, while negatively charged interfacial layers might attract these pro-oxidative ions (Mei, McClements, Wu, & Decker, 1998). We showed that FBPI had negative surface charges and MTG treatment further increased the net surface charges of FBPI (**Fig. 3A**). It is possible that increased negative charge by MTG treatment might bind more pro-oxidative metal ions, thus increasing protein oxidation.

Our results seemed to support the protective effect of protein against lipid oxidation. Tryptophan and cysteine are more oxidatively unstable than unsaturated fatty acids in emulsions (Elias, Kellerby, & Decker, 2008). Furthermore, amino acid residues such as

tryptophan, histidine, glutamic acid, aspartic acid, and phosphorylated serine and threonine are well known to have high affinities to metals and could act as metal chelators (Tong et al., 2000; Ueda, Gout, & Morganti, 2003).

4. Conclusions

This study demonstrated that FBPI in combination with MTG could serve as a good natural emulsifier in O/W emulsions. However, it is critical to control the degree of MTG modification. Prolonged MTG treatment (> 60 min) may induce excessive surface hydrophobicity and lead to droplets coalescence and accelerated protein oxidation. FBPI modified by MTG for 60 min could maintain physical stability by increasing the net surface charge. More importantly, it also could improve the stability of the emulsion toward lipid oxidation, which may be attributed to thicker interfacial layer around oil droplet, larger emulsion droplet size, and protective effect of protein.

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Figures

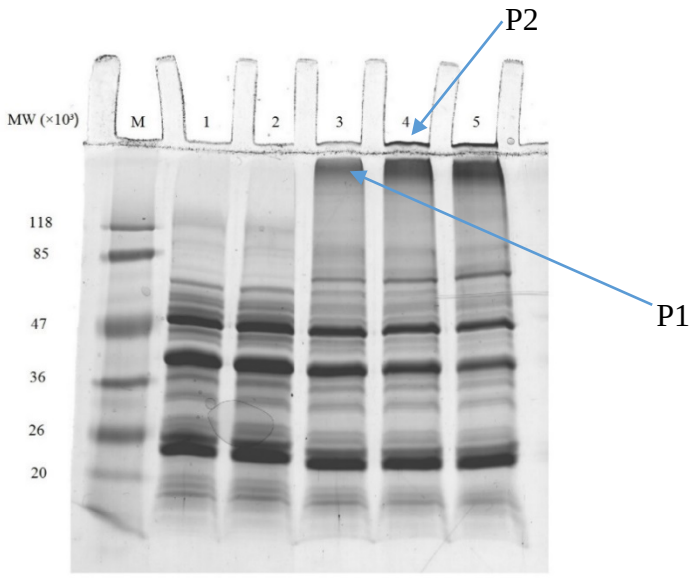

Fig. 1. SDS-PAGE patterns of native, control and MTG-FBPIs. Lanes: M = protein marker (in kDa); 1 = native-FBPI; 2 = control-FBPI; 3-5 = MTG60-FBPI, MTG120-FBPI and MTG240-FBPI. P1 and P2 were new polymeric species formed under MTG treatment. FBPI was incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI); FBPI incubated with NH_4Cl-inactivated MTG served as control-FBPI.

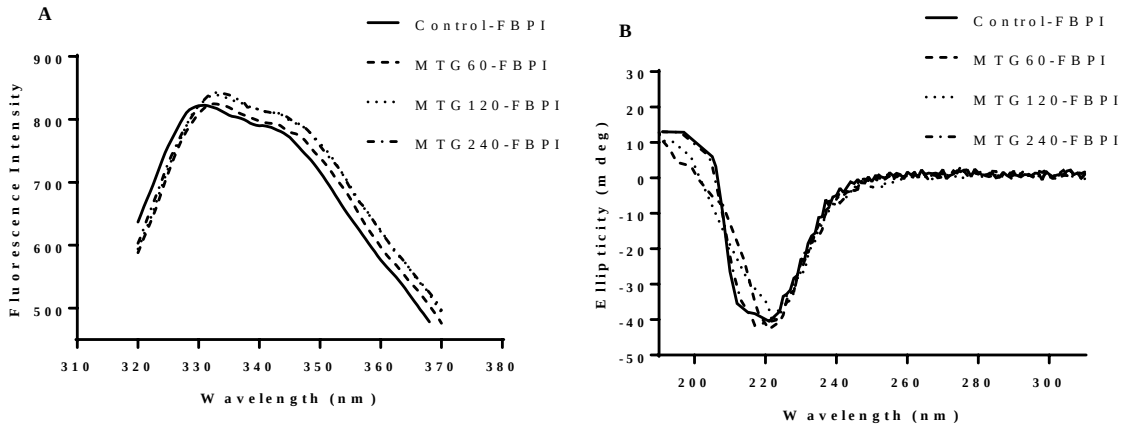

Fig. 2. Fluorescence spectra (A) and far-UV circular dichroism (FUCD) spectra (B) of control and MTG-FBPIs. FBPI was incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI); FBPI incubated with NH_4Cl-inactivated MTG served as control-FBPI.

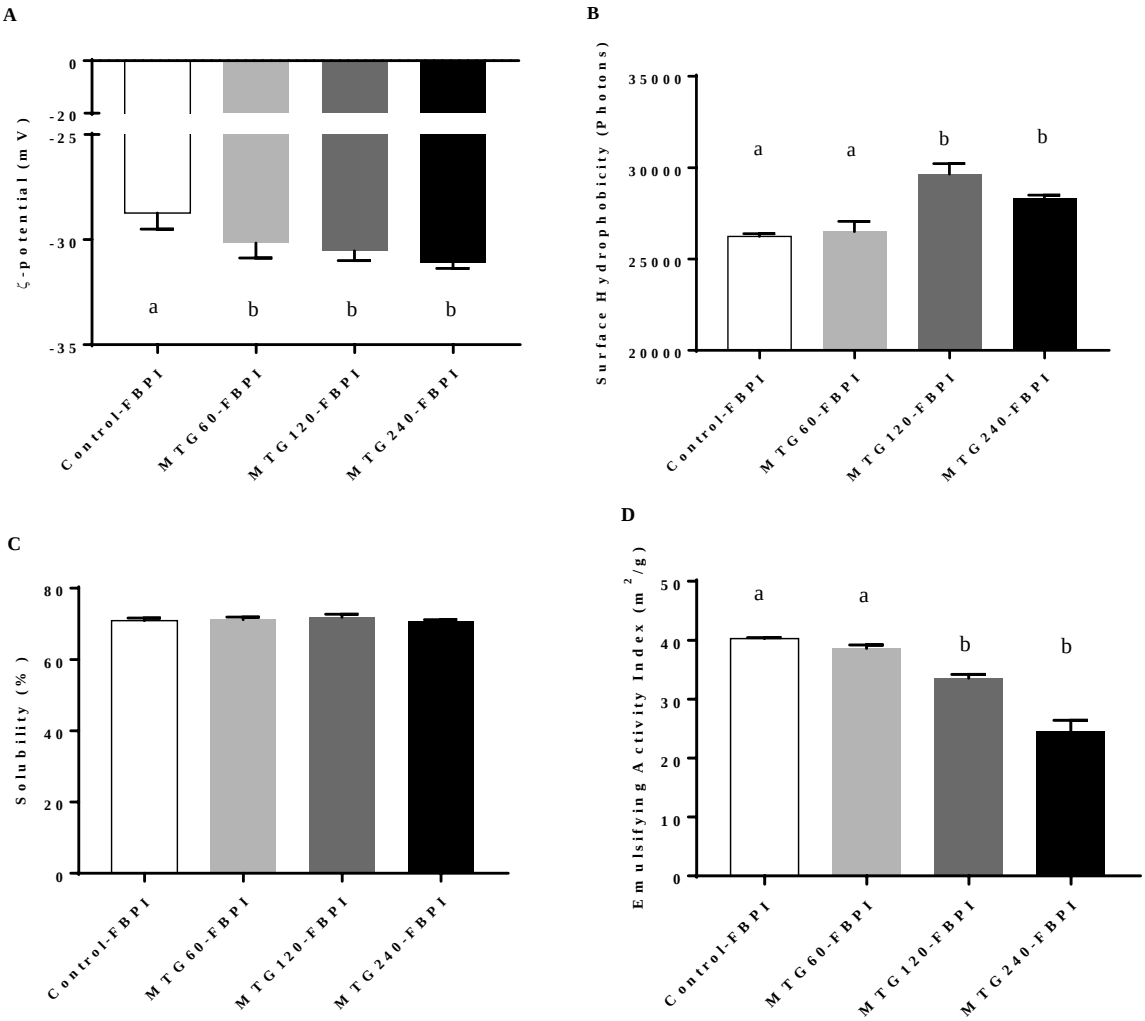

Fig. 3. Zeta-potential (A), surface hydrophobicity (B), solubility (C), and EAI (D) of control and MTG-FBPIs. FBPI was incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60-FBPI), 120 min (MTG120-FBPI), or 240 min (MTG240-FBPI); FBPI incubated with NH_4Cl-inactivated MTG served as control-FBPI. Significant differences ($p < 0.05$) were denoted by different letters.

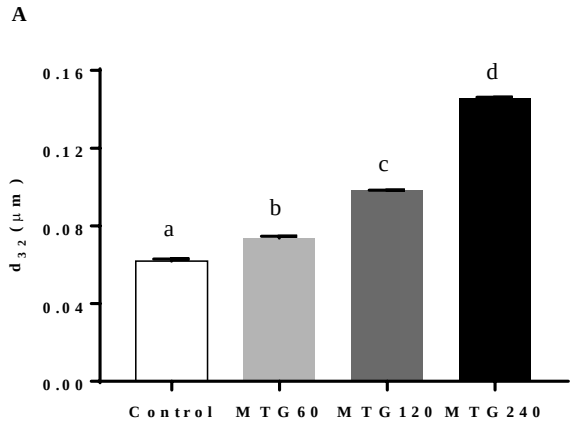

Fig. 4. The mean particle sizes of emulsions stabilized by control or MTG-FBPIs on day 0. Emulsions were stabilized with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control. Significant differences ($p < 0.05$) were denoted by different letters.

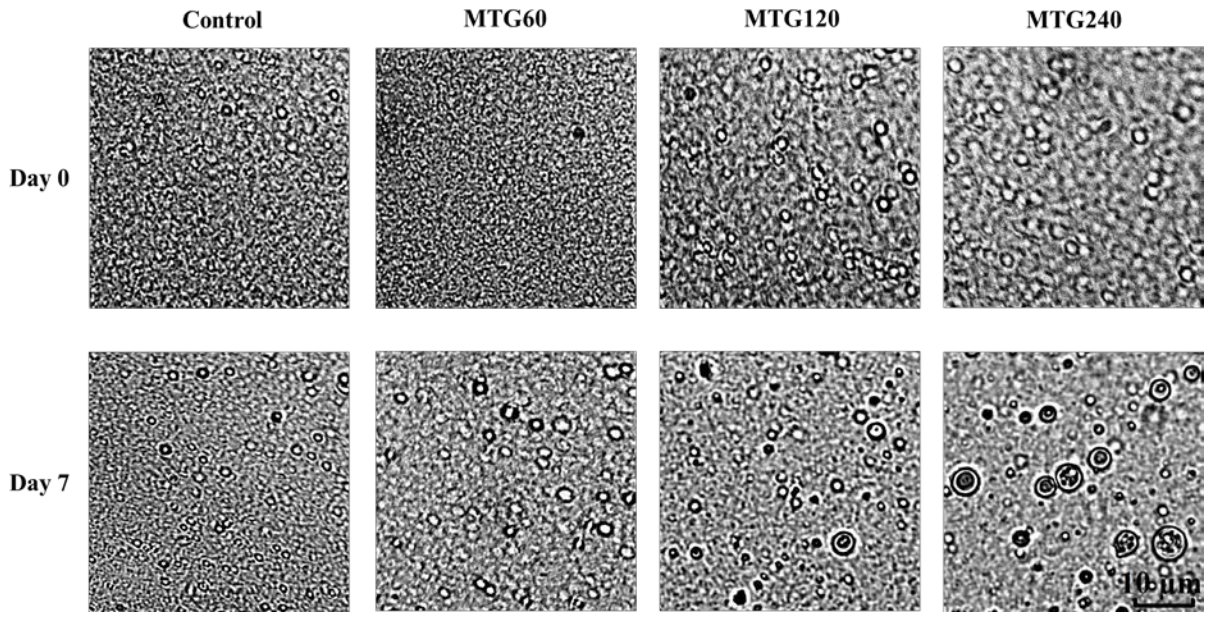

Fig. 5. The microscopic pictures of FBPI-stabilized emulsions at day 0 day 7. Emulsions were stabilized with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control.

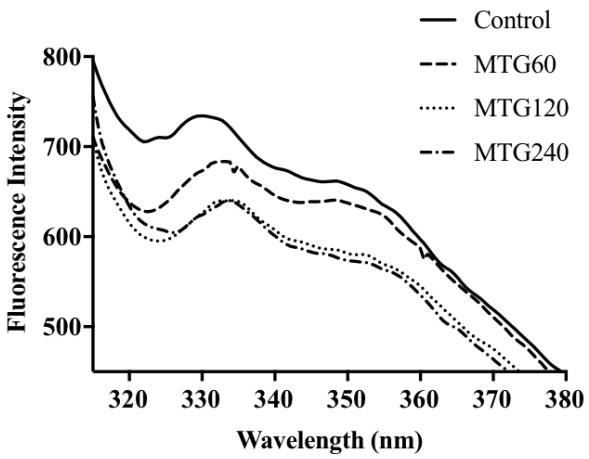

Fig. 6. Changes of tryptophan fluorescence intensities in emulsions incubated at 37 °C in dark with constant magnetic stirring over 7 days. Emulsions were stabilized with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control.

Table 1. Formation of conjugated dienes (CDs) and hexanal in emulsions stored at 37 °C in dark with constant magnetic stirring over 7 days.

Time	CDs (mmol/Kg)				Hexanal (peak area, × 10^4 AU)			
(day)	Control	MTG60	MTG120	MTG240	Control	MTG60	MTG120	MTG240
0	$2.2 \pm 0.1^{a\,A}$	$2.0 \pm 0.1^{a\,A}$	$1.8 \pm 0.1^{a\,A}$	$1.9 \pm 0.1^{a\,A}$	$1.1 \pm 0.3^{a\,A}$	$1.3 \pm 0.3^{a\,A}$	$1.1 \pm 0.2^{a\,A}$	$1.0 \pm 0.1^{a\,A}$
1	$3.1 \pm 0.1^{a\,B}$	$2.8 \pm 0.1^{a\,AB}$	$2.6 \pm 0.1^{a\,AB}$	$2.7 \pm 0.1^{a\,AB}$	$2.9 \pm 0.2^{a\,AB}$	$2.5 \pm 0.1^{a\,AB}$	$2.5 \pm 0.3^{a\,AB}$	$2.4 \pm 0.1^{a\,ABC}$
4	$6.9 \pm 0.1^{a\,C}$	$6.7 \pm 0.1^{a\,C}$	$6.5 \pm 1.3^{a\,C}$	$5.9 \pm 0.9^{a\,C}$	$30.2 \pm 4.6^{a\,C}$	$24.6 \pm 7.4^{a\,BC}$	$23.7 \pm 2.4^{a\,C}$	$26.1 \pm 4.4^{a\,AC}$
7	$11.7 \pm 0.3^{a\,D}$	$9.7 \pm 0.4^{b\,D}$	$10.1 \pm 1.2^{b\,C}$	$10.1 \pm 0.7^{b\,D}$	$133.3 \pm 10.5^{a\,D}$	$46.7 \pm 1.5^{b\,D}$	$50.1 \pm 5.3^{b\,D}$	$81.7 \pm 22.7^{b\,D}$

Emulsions were stabilized with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control. Data are expressed as mean ± standard deviation. Different lowercase letter indicates significant differences among treatment groups on each day, determined One-way ANOVA with Tukey's test ($p < 0.05$). Different uppercase letter indicates significant differences among different days within each treatment group, determined RM One-way ANOVA with Tukey's test ($p < 0.05$).

Table 2. Fluorescence of tryptophan and protein oxidation products (OPs) in emulsions stored at 37 °C in dark with constant magnetic stirring over 7 days.

Time	Tryptophan (fluorescence intensity)				OPs (fluorescence intensity)			
(day)	Control	MTG60	MTG120	MTG240	Control	MTG60	MTG120	MTG240
0	$909.2 \pm 3.4^{a\,A}$	$915.2 \pm 0.1^{a\,A}$	$921.5 \pm 1.7^{a\,A}$	$923.6 \pm 8.3^{a\,A}$	$106.9 \pm 0.7^{a\,A}$	$105.5 \pm 4.0^{a\,A}$	$105.2 \pm 0.8^{a\,A}$	$105.5 \pm 0.5^{a\,A}$
1	$906.4 \pm 6.8^{a\,AB}$	$887.2 \pm 9.2^{a\,B}$	$851.2 \pm 11.4^{b\,B}$	$864.5 \pm 10.7^{b\,B}$	$135.9 \pm 1.2^{a\,B}$	$140.4 \pm 5.7^{a\,B}$	$140.4 \pm 1.1^{a\,B}$	$138.2 \pm 0.5^{a\,B}$
4	$816.8 \pm 12.6^{a\,C}$	$806.1 \pm 4.6^{a\,C}$	$770.1 \pm 22.2^{b\,C}$	$763.8 \pm 16.1^{b\,C}$	$209.1 \pm 0.3^{a\,C}$	$214.1 \pm 10.1^{a\,C}$	$215.8 \pm 5.4^{a\,C}$	$218.6 \pm 3.6^{a\,C}$
7	$725.6 \pm 9.4^{a\,D}$	$659.6 \pm 22.6^{b\,D}$	$638.3 \pm 22.2^{b\,D}$	$636.5 \pm 8.7^{b\,D}$	$299.8 \pm 3.0^{a\,D}$	$331.3 \pm 16.8^{b\,D}$	$330.6 \pm 1.6^{b\,D}$	$311.3 \pm 0.1^{a\,D}$

Emulsions were stabilized with 3% FBPI that were incubated with MTG (5 U/g of protein substrate) at 37 °C for 60 min (MTG60), 120 min (MTG120), or 240 min (MTG240); emulsion stabilized with 3% FBPI that was incubated with NH_4Cl-inactivated MTG served as control. Data are expressed as mean ± standard deviation. Different lowercase letter indicates significant differences among treatment groups on each day, determined One-way ANOVA with Tukey's test ($p < 0.05$). Different uppercase letter indicates significant differences among different days within each treatment group, determined RM One-way ANOVA with Tukey's test ($p < 0.05$)